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Abstract: **OBJECTIVE:** Deficiency of pyruvate dehydrogenase complex (PDHC) is the most common genetic disorder leading to lactic acidosis. PDHC deficiency is genetically heterogeneous and most patients have defects in the X-linked E1- gene but defects in the other components of the complex encoded by PDHB, PDHX, DLAT, DLD genes or in the regulatory enzyme encoded by PDP1 have also been found. Phenylbutyrate enhances PDHC enzymatic activity in vitro and in vivo by increasing the proportion of unphosphorylated enzyme through inhibition of pyruvate dehydrogenase kinases and thus, has potential for therapy of patients with PDHC deficiency. In the present study, we investigated response to phenylbutyrate of multiple cell lines harboring all known gene defects resulting in PDHC deficiency. **METHODS:** Fibroblasts of patients with PDHC deficiency were studied for their enzyme activity at baseline and following phenylbutyrate incubation. Drug responses were correlated with genotypes and protein levels by Western blotting. **RESULTS:** Large deletions affecting PDHA1 that result in lack of detectable protein were unresponsive to phenylbutyrate, whereas increased PDHC activity was detected in most fibroblasts harboring PDHA1 missense mutations. Mutations affecting the R349- residue were directed to proteasome degradation and were consistently unresponsive to short-time drug incubation but longer incubation resulted in increased levels of enzyme activity and protein that may be due to an additional effect of phenylbutyrate as a molecular chaperone. **INTERPRETATION:** PDHC enzyme activity was enhanced by phenylbutyrate in cells harboring missense mutations in PDHB, PDHX, DLAT, DLD, and PDP1 genes. In the prospect of a clinical trial, the results of this study may allow prediction of in vivo response in patients with PDHC deficiency harboring a wide spectrum of molecular defects.

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RESEARCH ARTICLE

Phenylbutyrate increases pyruvate dehydrogenase complex activity in cells harboring a variety of defects

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Abstract

Objective: Deficiency of pyruvate dehydrogenase complex (PDHC) is the most common genetic disorder leading to lactic acidosis. PDHC deficiency is genetically heterogeneous and most patients have defects in the X-linked *E1-α* gene but defects in the other components of the complex encoded by *PDHB*, *PDHX*, *DLAT*, *DLD* genes or in the regulatory enzyme encoded by *PDP1* have also been found. Phenylbutyrate enhances PDHC enzymatic activity in vitro and in vivo by increasing the proportion of unphosphorylated enzyme through inhibition of pyruvate dehydrogenase kinases and thus, has potential for therapy of patients with PDHC deficiency. In the present study, we investigated response to phenylbutyrate of multiple cell lines harboring all known gene defects resulting in PDHC deficiency. **Methods:** Fibroblasts of patients with PDHC deficiency were studied for their enzyme activity at baseline and following phenylbutyrate incubation. Drug responses were correlated with genotypes and protein levels by Western blotting. **Results:** Large deletions affecting *PDHA1* that result in lack of detectable protein were unresponsive to phenylbutyrate, whereas increased PDHC activity was detected in most fibroblasts harboring *PDHA1* missense mutations. Mutations affecting the R349-α residue were directed to proteasome degradation and were consistently unresponsive to short-time drug incubation but longer incubation resulted in increased levels of enzyme activity and protein that may be due to an additional effect of phenylbutyrate as a molecular chaperone. **Interpretation:** PDHC enzyme activity was enhanced by phenylbutyrate in cells harboring missense mutations in *PDHB*, *PDHX*, *DLAT*, *DLD*, and *PDP1* genes. In the prospect of a clinical trial, the results of this study may allow prediction of in vivo response in patients with PDHC deficiency harboring a wide spectrum of molecular defects.

Introduction

Deficiency of nuclear-encoded pyruvate dehydrogenase complex (PDHC) is one of the most common inborn errors of mitochondrial energy metabolism.¹ PDHC catalyzes within mitochondria conversion of pyruvate to acetyl coenzyme A, a key regulatory step in the central pathway of energy production. This reaction is particularly important in brain which has obligatory requirement for aerobic glucose oxidation. Most patients with PDHC deficiency show progressive neurological degeneration and either persistent or episodic elevations of lactate in blood, cerebrospinal fluid (CSF), or both. Affected patients present with a graded spectrum of severity ranging from the most severe congenital brain malformations with overwhelming lactic acidemia and death in the neonatal period to development of Leigh syndrome or to a milder form with carbohydrate induced ataxia.^{1–3} PDHC is a multienzyme complex consisting of (1) thiamine diphosphate (ThDP)-dependent pyruvate dehydrogenase (E1), a heterotetrameric ($\alpha_2\beta_2$) protein; (2) dihydrolipoamide acetyltransferase (E2), which forms the structural core of the complex; and (3) FAD-containing dihydrolipoamide dehydrogenase (E3), which is integrated into the complex by an E3-binding protein (E3BP). PDHC is regulated through phosphorylation of E1- α subunit by four pyruvate dehydrogenase kinase (PDK) isoforms that inactivate the enzyme,⁴ whereas dephosphorylation by pyruvate dehydrogenase phosphatases (PDP1 and PDP2) restore PDHC activity.⁵ PDHC deficiency may be due to mutations in the genes *PDHA1*, *PDHB*, *PDHX*, *DLAT*, and *DLD* encoding for E1- α , E1- β , E3BP, E2, and E3, respectively, or to mutations of *PDP1* gene encoding pyruvate dehydrogenase phosphatase 1.^{1,2} The most common form is due to mutations of *PDHA1* gene located on the short arm of the X chromosome.⁶ We have previously shown that phenylbutyrate increases residual activity of PDHC by increasing the proportion of unphosphorylated enzyme and has potential for therapy of PDHC deficiency and systemic lactic acidosis.⁷ In the present study, to correlate in vitro drug response to the type of mutation, we investigated phenylbutyrate responsiveness on several cell lines harboring *PDHA1* mutations. In addition, we investigated phenylbutyrate response in patients' fibroblasts harboring defects in all remaining components of the multienzyme complex and in *PDP1* gene.

Material and Methods

PDHC activity was measured at baseline and after incubation with 1 mmol/L phenylbutyrate (Amonaps; Swedish Orphan International Lab, Stockholm, Sweden) in Dulbecco's Modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (FBS) for 24 h or 5 days on mitochondrial extracts prepared from synchronized fibroblasts (cultured in DMEM and 1% FBS for 24 h), as previously described.^{7,8} PDHC enzyme activity was calculated as nmol min⁻¹ mg⁻¹ of protein and expressed as fold increase of activity in cells treated with phenylbutyrate over untreated cells (at least $n = 2$ for each treatment). Statistical significance was computed using the Student's two-tailed test. $P < 0.05$ was considered statistically significant.

Fibroblasts were analyzed for expression of proteins of PDHC with the following primary monoclonal antibodies: anti-PDH-E1 α (9H9AF5), anti-PDH-E1 β (17A5E2H8), anti-PDH-E2 (15D3G9C11), anti-E3BP (13G2AE2BH5), and anti-PDH-E3 (EPR6635). The anti-PDH-E1 α , anti-PDH-E1 β , anti-PDH-E2, anti-E3BP mixed in a cocktail (#ab110416), and the anti-PDH-E3 (#ab133551) were purchased from Abcam (Cambridge, UK). The antibodies phosphodetect anti-PDH-E1 α (pSer²⁶⁴; AP1062), phosphodetect anti-PDH-E1 α (pSer²⁰³; AP1063), phosphodetect anti-PDH-E1 α (pSer²⁷¹; AP1064) were from Calbiochem (Darmstadt, Germany) and the anti-cytochrome c oxidase IV (COXIV; #4844) from Cell Signaling Technology Inc (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (ECL rabbit IgG, HRP-linked whole antibody from donkey [GE Healthcare, Milan, Italy NA934] and ECL anti-mouse IgG, HRP-linked species-specific whole antibody from sheep [GE Healthcare, NA931]) were diluted in 5% dried skim milk and in 1% bovine serum albumin (BSA) in tris-buffered saline-Tween20. Protein bands were visualized with a chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and band intensities were quantified with Quantity One-4.6.7 Basic (1-D Analysis Software; Bio-Rad Laboratories Inc. Hercules, CA, USA).

For proteasome inhibition, cells were cultured in DMEM supplemented with 10% FBS and after the 5-day treatment with 1 mmol/L of phenylbutyrate, were incubated with 40 μ mol/L MG132 (Selleckchem, Munich, Germany) or Dimethyl Sulfoxide (DMSO) for 6 h, washed once with cold phosphate-buffered saline (PBS), and scraped with RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.1% SDS) containing complete protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were incubated for 20 min at 4°C, centrifuged at 16,000 g for 10 min and cell lysates were used for Western blot.

Results

We analyzed PDHC activity in untreated and phenylbutyrate-treated skin fibroblasts from patients harboring all

Table 1. PDHC-deficient fibroblasts.

Cell line	Gender	Enzyme activity (% mean control)	Allele 1 nucleotide (protein) ^a	Allele 2 nucleotide (protein) ^a
<i>PDHA1 mutations</i>				
1 ^b	Male	62%	c.749C>T (p.P221L)	—
2 ^c	Male	46%	c.787C>G (p.R234G)	—
3 ^b	Male	56%	c.832G>C (p.G249R)	—
4 ^c	Male	21%	c.1132G>C (p.R349C)	—
5 ^b	Male	45%	c.1132C>T (p.R349C)	—
6 ^b	Male	7%	c.1133G>A (p.R349H)	—
7 ^b	Male	18%	c.1133G>A (p.R349H)	—
8 ^d	Male	52%	c.483C>T (p.Y132Y)	—
9 ^b	Male	7%	c.1050_1133dup84 (p.Q351_R378dup)	—
10	Female	47%	c.302G>T (p.C72F)	wt
11 ^b	Female	48%	c.302G>T (p.C72F)	wt
12 ^b	Female	30%	c.355C>T (p.R90W)	wt
13 ^b	Female	23%	c.379C>T (p.R98W)	wt
14 ^b	Female	83%	c.787C>G (p.R234G)	wt
15	Female	13%	c.904C>T (p.R273C)	wt
16 ^b	Female	21%	c.904C>T (p.R273C)	wt
17 ^b	Female	19%	c.904C>T (p.R273C)	wt
18	Female	57%	c.924G>T (p.Q279H)	wt
19 ^b	Female	35%	c.498C>T (p.I137I)	wt
20 ^b	Female	8%	c.292-2A>G (p.E98_Q170del)	wt
21	Female	38%	c.1140_1162del (p.N381SfsX43)	wt
22	Female	22%	c.934_940del (p.S312VfsX12)	wt
23 ^b	Female	19%	2.14 Mb deletion	wt
<i>PDHB mutations</i>				
24 ^b	Male	75%	c.301A>G (p.M101V)	c.301A>G (p.M101V)
25 ^b	Male	70%	c.301A>G (p.M101V)	c.793-2A>G (p.I266LfsX26)

(Continued)

Table 1. Continued.

Cell line	Gender	Enzyme activity (% mean control)	Allele 1 nucleotide (protein) ^a	Allele 2 nucleotide (protein) ^a
26 ^b	Female	55%	c.301A>G (p.M101V)	c.301A>G (p.M101V)
27	Female	26%	c.497A>G (p.Y137C)	c.497A>G (p.Y137C)
<i>PDHX mutations</i>				
28 ^b	Male	25%	c.1426C>T (p.R476X)	c.1426C>T (p.R476X)
29 ^b	Male	41%	c.1182+2T>C (p.I386SfsX13)	c.1182+2T>C (p.I386SfsX13)
30 ^b	Male	35%	c.160+1G>A (p.G27X)	c.965-1G>A (p.D322AfsX6)
31 ^b	Female	25%	c.620delC (p.P207LfsX16)	c.620delC (p.P207LfsX16)
32	Male	14%	Exon 4 and 5 deletion	Exon 4 and 5 deletion
<i>DLAT mutations</i>				
33	Female	13%	c.1624-1626del (p.V560del)	ND
<i>DLD mutations</i>				
34 ^b	Male	28%	c.858dup (p.G287WfsX4)	c.1174G>A (p.V392M)
35 ^e	Male	27%	c.105insA (p.Y35X)	c.685G>T (p.G229C)
<i>PDP1 mutations</i>				
36 ^f	Male	33%	c.851_853del (p.L284del)	c.851_853del (p.L284del)

wt, wild-type; ND, not determined.

^aNucleotides are reported according to NCBI gene sequences (NM_000284.3 for *PDHA1*, NM_000925.3 for *PDHB*, NM_003477.2 for *PDHX*, NM_001931.4 for *DLAT*, NM_000108.3 for *DLD*, NM_001161781 for *PDP1*). The amino acid positions of the mutations are reported considering the mature NCBI protein sequences (NP_000275.1 for *PDHA1* [361 amino acids], NP_000916.2 for *PDHB* [329 amino acids], NP_003468.2 for *PDHX* [448 amino acids], NP_001922.2 for *DLAT* [561 amino acids], NP_060914.2 for *DLD* [474 amino acids], NP_001155253.1 for *PDP1* [466 amino acids]) after signal peptide cleavage. Patients previously described by ^bImbard et al.¹², ^cDeBrosse et al.³, ^dBoichard et al.¹⁰, ^eShag et al.²⁸, and ^fMaj et al.²⁹.

known molecular defects responsible for PDHC deficiency, that are, mutations in *PDHA1*, *PDHB*, *PDHX*, *DLAT*, *DLD*, and *PDP1* genes (Table 1). As a control, enzyme activity was measured at baseline and after phenylbutyrate in fibroblasts from a control subject. Among 16 cell lines with *PDHA1* missense mutations (Table 1), 11 cell lines showed a significant increase in enzyme activity following incubation with phenylbutyrate and had detectable E1- α protein by Western blotting (Fig. 1A and B). Fibroblasts from nine male patients harboring missense (p.P221L, p.R234G, p.G249R, p.R349C, p.R349H),

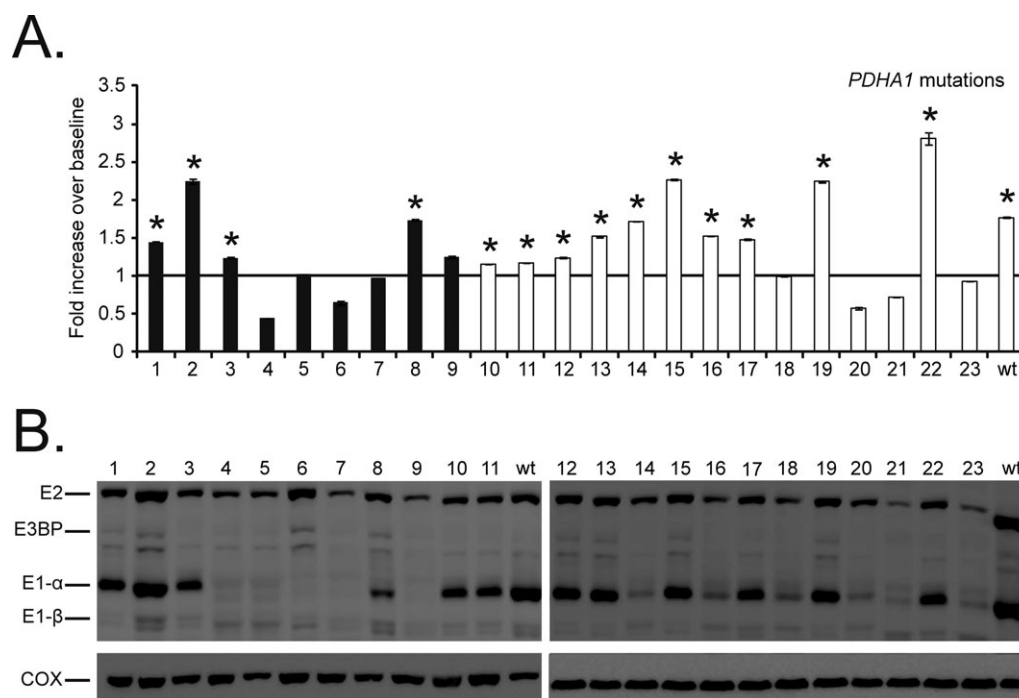


Figure 1. (A) PDHC activity in fibroblasts with *PDHA1* mutations. PDHC activity is shown as fold increase in baseline activity after incubation with phenylbutyrate. Bars indicate average \pm standard error of the mean; * $P < 0.05$. PDHC activity was increased in four of the nine fibroblast cell lines from male patients shown as black bars and in 10 of the 14 fibroblast cell lines from female patients shown as white bars. (B) Western blotting of skin fibroblasts with *PDHA1* mutations with a cocktail of antibodies recognizing E1- α (43 kDa), E1- β (39 kDa), E2 (69 kDa), and E3BP (54 kDa) proteins. Western blotting for cytochrome c oxidase (COX; 17 kDa) was performed as mitochondrial marker. The *PDHA1* mutations of the corresponding patient cell lines are shown in Table 1. wt, wild-type control fibroblasts.

splicing (c.483C>T), or truncating (p.Q351_R378dup) mutations were studied (Table 1, black bars in Fig. 1A). Hemizygous cell lines from patients carrying missense mutations p.P221L, p.R234G, and p.G249R (cell lines 1, 2, and 3) showed elevated PDHC activities (62%, 46%, and 56% of control mean), detectable E1- α protein by Western blotting, and were all responsive to phenylbutyrate (Fig. 1A and B, and Table 1). Four cell lines not responsive to phenylbutyrate all carried *PDHA1* missense mutations affecting the R349- α residue, had relatively lower levels of PDHC residual activity ranging from 7% to 45%, and showed very low-to-undetectable levels of protein E1- α by Western blotting (cell lines 4–7, Fig. 1A, and Table 1). Lack of increase in PDHC activity following phenylbutyrate incubation was also previously observed in another cell line (designated as Pt 3) from an unrelated patient also harboring a mutation affecting R349- α .⁷

It was previously shown that cells harboring the p.R349C mutation were also unresponsive to short-term incubation with dichloroacetate (DCA), a pyruvate analog which increases PDHC activity.⁹ In contrast, sustained DCA incubation reduced turnover of E1- α protein and increased PDHC activity.⁹ Therefore, we investigated longer incubation with phenylbutyrate in fibroblasts har-

boring mutations affecting R349- α (cell lines 4–7 and Pt 3⁷) and found that 5-day drug incubation increased PDHC enzyme activity (Fig. 2A). Following 5-days of incubation with phenylbutyrate, we also observed an increase in the amount of E1- α protein compared to untreated cells (Fig. 2B). The ubiquitin-proteasome is the major route for degradation of cell proteins and thus, we investigated E1- α protein levels in fibroblast cell line 5, carrying the p.R349C mutation, by inhibiting proteasome activity with the MG132 proteasome inhibitor. Incubation with MG132 increased E1- α protein to levels similar to those observed in phenylbutyrate-treated cells but no further increase in E1- α was observed in cells cotreated with phenylbutyrate and MG132 (Fig. 2B). Taken together, these results may indicate that E1- α harboring the p.R349C mutation is degraded by the proteasome and phenylbutyrate increases resistance of the mutated protein to proteasome degradation.

The synonymous mutation c.483C>T (p.Y132Y) of cell line 8 causes aberrant splicing in 40% of total cDNA with exon 5 skipping predicted to result in a frameshift and premature termination after the addition of 10 aberrant amino acids (p.Arg141AlafsX11).¹⁰ Immunoblotting of these fibroblasts resulted in detectable E1- α protein band

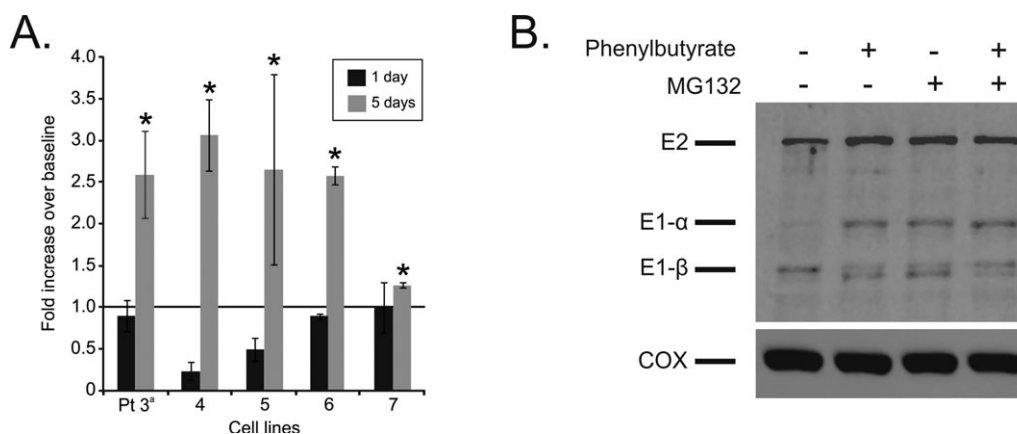


Figure 2. (A) PDHC activity in fibroblasts with mutations affecting R349 residue of E1- α protein following shorter (1 day) and longer (5 days) phenylbutyrate incubations. (B) Western blotting for E1- α (43 kDa), E1- β (39 kDa), and E2 (69 kDa) protein of cell line 5 harboring p.R349C mutation incubated with either vehicle, phenylbutyrate, MG132, or a combination of phenylbutyrate and MG132. Cytochrome c oxidase (COX; 17 kDa) was used as mitochondrial control marker.

of normal size expressed from the proportion of correctly spliced mRNA (Fig. 1A).¹⁰ Phenylbutyrate likely increased enzyme activity of normal E1- α enzyme expressed from the correctly spliced mRNA. The hemizygous cell line 9 from male patient carrying the c.1050_1133dup84 resulted in very low PDHC activity, in no detectable E1- α protein, and phenylbutyrate failed to increase significantly PDHC activity (Fig. 1A and B).

Fibroblasts from 14 female patients harboring missense (p.C72F, p.R90W, p.R98W, p.R234G, p.R273C, p.Q279H), splicing (c.498C>T), or truncating (p.E98_Q170del, p.S312VfsX12, p.N381SfsX43, 2.15 Mb deletion) mutations were studied (Table 1, white bars in Fig. 1A). Cell lines with *PDHA1* mutations from female patients are expected to express both wild-type and mutant alleles.⁶ Furthermore, skewed X-inactivation is found in approximately one fourth of skin fibroblasts from PDHC-deficient patients.⁶ Thus, PDHC activity in cells from female patients results from both residual activity of the enzyme encoded by the mutant allele and the activity of the enzyme expressed from the wild-type allele. Phenylbutyrate increases PDHC activity in wild-type cells (Fig. 1A)⁷ and thus, it is not possible to determine whether phenylbutyrate increases the activity of the enzyme encoded by the wild-type or by the mutated allele in cells from female patient. Nine fibroblast cell lines with missense mutations resulted in variable PDHC activities ranging from 13% to 83%, detectable levels of E1- α protein, and were all responsive to phenylbutyrate incubation (Table 1, white bars in Fig. 1A and B). The p.R273C mutation present in cell lines 15–17 results in normal levels of E1- α immunoreactive protein and catalytically inactive enzyme¹¹ and thus, phenylbutyrate is likely increasing enzyme activity of E1- α expressed from the wild-type

allele. Cell line harboring the p.Q279H mutation showed low levels of E1- α protein by Western blotting and was unresponsive to phenylbutyrate (cell line 18, Fig. 1A and B, Table 1).

The synonymous mutation c.498C>T (p.I137I) in cell line 19 from a female patient causes aberrant splicing in 40% of total cDNA with exon 5 skipping predicted to result in a frameshift with premature termination after the addition of 10 aberrant amino acids (p.Arg141AfsX11).¹⁰ This mutation results in detectable E1- α protein by Western blotting of skin fibroblasts (Fig. 1B). This protein band is likely a combination of the products of the wild-type allele and the correctly spliced protein from the allele carrying the mutation. Likely, phenylbutyrate is increasing the activity of the enzyme expressed from the wild-type allele and from the proportion of enzyme derived from the correctly spliced mRNA expressed from the mutant allele (Fig. 1A). Cell line 22 from a female patient harboring the mutation c.934_940del and with 22% of residual enzyme activity had a significant 2.8-fold increase in PDHC activity after incubation with phenylbutyrate and showed detectable levels of E1- α by Western blotting (Table 1, Fig. 1A and B). Three cell lines of fibroblasts harboring splicing mutation or deletions (fibroblast cell lines 20, 21, and 23) carrying the mutations c.292-2A>G, c.1140_1162del, and a 2.14 Mb deletion, respectively, showed low-to-undetectable levels of E1- α protein and low PDHC activity (8–38%) and were unresponsive to phenylbutyrate (Fig. 1A and B). The absence or the low levels of E1- α protein by Western blotting suggests occurrence in these cell lines of skewed X-inactivation favoring the mutant allele that could explain the lack of response to phenylbutyrate.

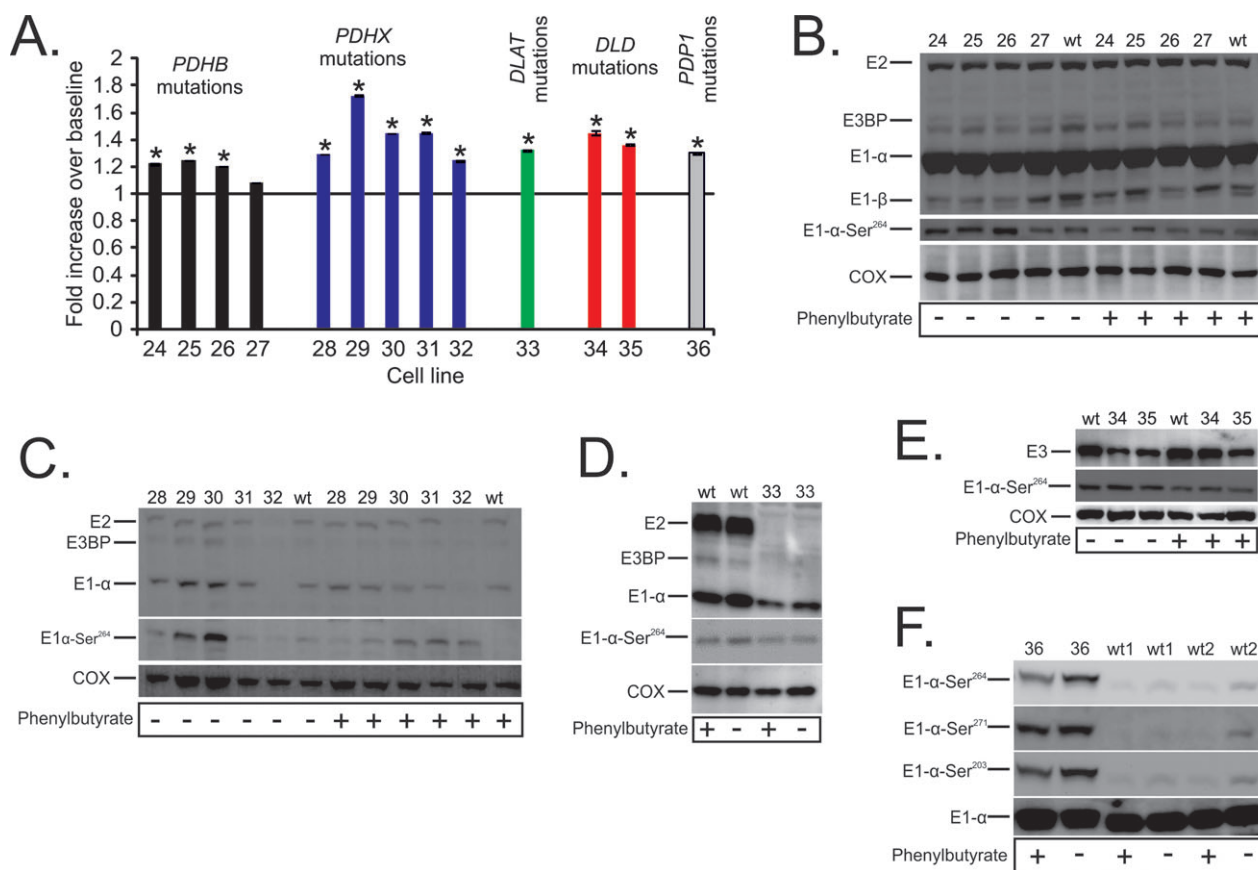


Figure 3. (A) PDHC activity in fibroblasts with *PDHB*, *PDHX*, *DLD*, or *PDP1* mutations. PDHC activity is shown as fold increase in baseline activity following incubation with phenylbutyrate. Bars indicate average \pm standard error of the mean; * $P < 0.05$. Western blotting for E1- α (43 kDa), E1- β (39 kDa), E2 (69 kDa), and E3BP (54 kDa) proteins of skin fibroblasts with *PDHB* (B), *PDHX* (C), or *DLAT* (D) mutations. (E) Western blotting for E3 protein of cell lines 34 and 35 harboring *DLD* mutations. PDHC phosphorylation was evaluated with an antibody against the phosphorylated E1- α (E1- α -Ser²⁶⁴). Cytochrome c oxidase (COX; 17 kDa) was used as mitochondrial marker. (F) Western blotting with antibodies against the three phosphorylated sites of E1- α or total E1- α in fibroblast cell line 36 harboring a homozygous *PDP1* mutation. wt1, control 1; wt2, control 2. The *PDHB*, *PDHX*, *DLD*, or *PDP1* mutations for each cell lines are shown in Table 1.

So far, 12 *PDHB* mutations have been reported and four mutations, including the p.M101V found in patients of North African descent,¹² clustered in the same region.^{12–14} We investigated phenylbutyrate in four cell lines from patients harboring *PDHB* mutations (cell lines 24–27, Table 1) including the p.M101V mutation, and three of them showed a statistically significant increase in PDHC activity after phenylbutyrate incubation (Fig. 3A). Three of these cell lines with *PDHB* mutations showed a reduction in E1- β protein by Western blotting, whereas the nonresponder cell line (cell 27) had E1- β levels similar to wild-type levels (Fig. 3B). In two of the three cell lines responding to phenylbutyrate in terms of increased enzyme activity (cell lines 24 and 25), an increase in E1- β protein was observed by Western blotting after phenylbutyrate incubation (Fig. 3B). In the three cell lines that responded to phenylbutyrate in terms of increased PDHC

activity, a reduction in phosphorylated E1- α levels was observed, whereas cell line 27 that failed to respond to phenylbutyrate did not show a reduction in phosphorylated E1- α levels (Figs. 3B, S1).

The E3BP lacks catalytic activity and has a structural role in the formation of the enzyme complex binding E3 to E2. Mutations in *PDHX* gene encoding E3BP are the second most common cause of PDHC deficiency^{2,12} and the vast majority of the mutations are aberrant splicing or nonsense mutations.¹² In this study, we investigated five cell lines harboring mutations in *PDHX* gene that all carried nonsense mutations or deletions and showed enzyme activity ranging from 14% to 41% (cell lines 28–32, Table 1, Fig. 3A and C). Two of the cell lines (31 and 32, Table 1) harboring a single nucleotide deletion leading to frameshift or an exon 4 and 5 deletion did not show detectable E3BP by Western blotting (Fig. 3C). All five

cell lines were found to be responsive to phenylbutyrate with an average 1.4-fold increase in enzyme activity (Fig. 3A). Phenylbutyrate incubation did not appear to affect E3BP protein levels determined by Western blotting and a reduction in phosphorylated E1 α levels was observed in four of six cell lines (Figs. 3E, S1).

One cell line with *DLAT* mutation was available for analysis and a 1.3-fold increase enzyme activity was detected in this cell line after incubation with phenylbutyrate (cell line 33, Table 1 and Fig. 3A). Fibroblasts of cell line 33 had low enzyme activity and did not show detectable levels of E2 protein by Western blotting even after incubation with phenylbutyrate (Fig. 3D). Only one mutation was detected in this patient. It appears that mutations in the gene encoding the E2 are rare cause of PDHC deficiency.^{2,15–17} One patient previously reported had undetectable E2 immunoreactive protein,¹⁶ whereas two other patients were found to express reduced but detectable levels of E2 protein.¹⁷ The clinical presentation appeared to be more severe in the patient with undetectable E2 protein compared to the patients with detectable E2 that had relatively mild symptoms and episodic dystonia. DCA was previously shown to increase PDHC activity in fibroblasts of these patients with *DLAT* mutations.¹⁷ Phenylbutyrate was also found to correct the morphological, locomotor, and behavioral phenotype of the *noa*^{m631} zebrafish model carrying a missense *DLAT* gene mutation.⁷ Thus, E2 defects resulting from *DLAT* mutations appear to be responsive to phenylbutyrate. A reduction in phosphorylated E1 α levels was observed in this cell line with *DLAT* mutation following phenylbutyrate incubation (Figs. 1, 3B).

We also investigated phenylbutyrate in two cell lines harboring *DLD* mutations resulting in E3 deficiency and both showed an average 1.4-fold increase in baseline PDHC activity (cell lines 34 and 35, Table 1 and Fig. 3A). Both cell lines expressed the E3 protein, as shown by Western blotting with anti-E3 antibody (Fig. 3E). Clinical and biochemical presentation of this disorder is different from the common presentation of other PDHC defects.^{18,19} E3 is a common component of all three mitochondrial multienzyme complexes, and *DLD* mutations result in a disorder associated with a combination of features of branched chain α -ketoacid dehydrogenase (BCKDC), PDHC, and α -ketoglutarate dehydrogenase complex (KGDC) deficiencies. Phenylbutyrate inhibits the kinase of BCKDC (BDK) and PDKs^{7,20} and thus, it is expected to result in improvements of both BCKDC and PDHC activities. Like cell lines with other defects, a reduction in phosphorylated E1 α levels was observed in cells with *DLD* mutations (Figs. 3B, S1).

Finally, we investigated one cell line with *PDPI* mutations that was found to respond to phenylbutyrate (cell

line 36, Table 1; Fig. 3A). Phosphorylation of E1- α occurs at three serine residues (Ser264- α , site 1; Ser271- α , site 2; and Ser203- α , site 3). As expected based on the deficiency of the phosphatase activity, the amount of phosphorylated E1- α in this cell line is increased compared to control wild-type cell lines (Fig. 3F). Following phenylbutyrate incubation, phosphorylated E1- α was significantly reduced at all three phosphorylation sites (Fig. 3F) that is consistent with the mechanism of action of phenylbutyrate based on PDK inhibition.⁷

Discussion

Phenylbutyrate enhances PDHC activity in vitro and in vivo by increasing the proportion of unphosphorylated enzyme through inhibition of PDK. It also reduces lactic acid in a mouse model of systemic lactic acidosis, and rescues the phenotype of a zebrafish PDHC-deficient disease model.⁷ In the present study, we investigated the efficacy of phenylbutyrate in PDHC-deficient cells harboring a wide spectrum of genetic defects and we correlated genotypes with drug response. We found that phenylbutyrate increases PDHC activity in the majority of analyzed fibroblasts. Increased PDHC activity was detected in most of the fibroblasts with *PDHA1* missense mutations. Large deletions or nonsense *PDHA1* mutations resulting in no detectable protein by Western blotting were in general not responsive to phenylbutyrate. The gene encoding E1- α is located on the X chromosome, yet males and females are affected in approximately equal numbers due to the high proportion of heterozygous females manifesting the disease. All male patients with *PDHA1* mutations have some degree of residual activity, whereas several female patients have null mutations resulting in complete deficiency of the enzyme subunit. It appears that complete PDHC deficiency is incompatible with normal development in hemizygous males but can be tolerated in heterozygous females due to the proportion of cells expressing the normal X chromosome. In cells from female patients, due to skewed pattern of X-inactivation favoring the normal X chromosome, enzyme activity might be higher, even falling within the normal range. In this context, the great majority of mRNA molecules arise from the normal gene. Phenylbutyrate increases PDHC activity in wild-type cells⁷ and thus, it is not possible to determine in cells from female patients whether phenylbutyrate increases the activity of the enzyme encoded by the wild-type or by the mutated allele. Nevertheless, we cannot rule out that even an increase in PDHC activity in cells expressing the normal allele might be beneficial in patients with PDHC deficiency.

Cells harboring mutations affecting the R349- α residue of the E1- α protein were consistently found to be unresponsive to short-term incubation with phenylbutyrate

but longer incubations resulted in increased activity. These results indicate that in addition to the direct effect on PDK,⁷ phenylbutyrate increases the stability of E1- α protein, at least in cells harboring missense mutations affecting the R349- α . This result is consistent with the previously recognized function of phenylbutyrate as chemical chaperone²¹ and with reduced E1- α protein turnover observed with sustained DCA incubation in addition to PDK inhibition by DCA.^{9,22,23}

We found that mutations affecting *PDHB*, *PDHX*, *DLAT*, and *DLD* genes were also responsive, thus potentially broadening therapeutic applications of phenylbutyrate to patients that harbor all known defects of PDHC deficiency. In these cell lines a reduction in phosphorylated E1- α protein levels was observed by Western blotting, thus suggesting that like cells with *PDHA1* mutations, the increase in enzyme activity in cells harboring these defects is dependent, at least in part, on changes of phosphorylation status of the enzyme complex induced by phenylbutyrate.

Similar to phenylbutyrate, DCA also increases PDHC activity and is effective in reducing blood, CSF, and brain lactate.²⁴ However, in a controlled trial in children with various forms of congenital lactic acidosis, chronic DCA administration did not result in improvements of neurologic problems and other clinical outcomes.²⁵ Moreover, DCA has raised some concerns because it has been associated with hepatocellular and peripheral nerve toxicity, particularly in adults.²⁶ In contrast, phenylbutyrate is a drug already approved for use in humans, its safety profile is well known,²⁷ and thus it is attractive for therapy of patients with PDHC deficiency.

Enzyme activity measured in skin fibroblasts exhibit large variations among PDHC-deficient patients and does not correlate with the predicted severity of the mutation and cognitive outcomes, either in males or females.³ Nevertheless, enzyme activity appears to correlate with survival at least in males with *PDHA1* mutations³ and thus, drug interventions aiming at increasing enzyme residual activity have potential for achieving clinically relevant results. However, it is unlikely that such drug interventions will reverse pre-existing severe brain damage, such as Leigh syndrome.

In summary, cells harboring *PDHA1* gene mutations, that are the most common defects accounting for 80–90% of cases of PDHC deficiency with known mutations,^{2,3,12} respond to phenylbutyrate, especially if they carry missense mutations, have PDHC activity >10%, or exhibit detectable protein by Western blotting. Therefore, patients carrying these defects are candidates for such drug intervention. Cell lines from patients carrying other less common molecular defects also appear to be responsive to phenylbutyrate.

In conclusion, the results of the present study may help to predict whether a patient with PDHC deficiency will be responsive to phenylbutyrate therapy based on the affected gene, type, and location of the mutation.

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Authors Contributions

R. Ferriero performed all experiments included in the study. A. Boutron, M. Brivet, D. Kerr, E. Morava, R. J. Rodenburg, L. Bonafé, M. R. Baumgartner, Y. Anikster, and N. E. Braverman provided fibroblast cell lines from PDHC-deficient patients with molecular diagnoses. N. Brunetti-Pierri designed and supervised the study.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Average intensities of Western blotting bands of phosphorylated E1- α normalized for total E1- α from two independent experiments of fibroblasts with *PDHB*, *PDHX*, *DLAT*, and *DLD* mutations (Table 1) incubated with phenylbutyrate or vehicle. * $P < 0.05$.